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CHD8, A Novel Beta-Catenin Associated Chromatin Remodeling Enzyme, Regulates Androgen Receptor Mediated Gene Transcription

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14. ABSTRACT

The activity of the androgen receptor (AR) is critical for normal prostate development and function, but AR activity also plays a major role in the development and progression of prostate cancer. To better understand the function of beta-catenin in AR mediated transcription, we have identified a novel chromatin remodeling enzyme, CHD8, that can associate with beta-catenin and functions in AR mediated gene transcription. Year 1 was focused on the first specific aim of this proposal; the interaction of CHD8 with beta-catenin and the androgen receptor. Experiments were performed to characterize this interaction in vitro using recombinant proteins, and reagents were made to characterize this interaction in vivo using chromatin immunoprecipitation techniques. This work is required for the study of the function of CHD8 and β-catenin in AR mediated transcription and the ultimate understanding of the role played by CHD8 in prostate cancer progression.

15. SUBJECT TERMS

None provided.

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INTRODUCTION

The activity of the androgen receptor (AR) is critical for normal prostate development and function, but AR activity also plays a major role in the development and progression of prostate cancer. Current therapies utilize this fact and are aimed at reducing serum androgens and therefore, inhibiting AR. While these therapies show initial success in preventing tumor growth, they can ultimately fail due to the development of an androgen independent disease. β-catenin, a known oncogene, has been shown to directly interact with AR and also to function as a transcriptional coactivator for AR. To better understand the function of β-catenin in AR mediated transcription, we have identified a novel chromatin remodeling enzyme, CHD8, that can associate with β-catenin and functions in AR mediated gene transcription. The identification and characterization of novel factors, such as CHD8, that can function as coactivators of AR mediated transcription could result in the discovery of novel diagnostic, preventative, or therapeutic targets for prostate cancer.

BODY

β-catenin, a known oncogene, functions as a transcriptional coactivator for AR through a direct protein/protein interaction (1-5). Although the significance of this interaction is not known, understanding the crosstalk between these oncogenic pathways is critical to delimitating the function of AR in tumorigenesis. To better understand the function of β-catenin in AR mediated transcription, we have identified novel chromatin remodeling enzyme, CHD8 that can associate with β-catenin and functions in AR mediated gene transcription. **The hypothesis of this proposal is that through a direct interaction with β-catenin, CHD8 is directed to androgen responsive promoters and functions in regulating transcription by altering the chromatin structure.** This hypothesis will be tested by addressing the following Aims: 1) Does β-catenin target CHD8 to AR responsive promoters? 2) Are CHD8 and β-catenin both required to affect AR transcriptional activity? 3) Is CHD8 required for prostate cancer progression? The proposed studies will provide detailed information concerning the role of β-catenin and CHD8 in AR mediated transcription. This information will be valuable for the understanding of how β-catenin mediates AR transcriptional activation, and also will provide general insight into the mechanisms of prostate neoplastic transformation through the control of chromatin structure.

Year 1. The majority of the work proposed in months 1-12 addressed the first Aim by analyzing the interaction of CHD8 and β-catenin with the androgen receptor. Initial focus was placed on the production and optimization of recombinant proteins for protein/protein interaction studies (Task 1a). The recombinant proteins needed were CHD8, AR and β-catenin. As shown in the preliminary studies, full length CHD8 was successfully produced using a baculovirus expression system. Attempts were made at producing AR, but ultimately recombinant baculovirus for the expression of AR was obtained from Dr. James Dalton at Ohio State University (6). After significant optimization, expression conditions were identified for the production of full length 6-His tagged AR. Constructs were also generated for the production of full length β-catenin in a baculovirus expression system. Expression conditions were optimized, and full length β-catenin was also produced. A construct for the expression and purification of GST-tagged AR was obtained from the laboratory of our collaborator Dr. D. Robins. This construct was used as a

basis for the creation of various AR-GST fusions including a GST-N-terminal fragment, a GST-DBD-fragment, and a GST-LBD-fragment. Optimization and possible redesigning of these fragments is necessary as the GST-DBD-fragment fails to express detectable protein, and the GST-N-terminal fragment and GST-LBD-fragment produce multiple fragments that are essentially unusable in protein/protein interaction experiment. Several different fragments with alternate boundaries have been created and are currently being tested. The production of recombinant proteins must sometimes be designed and optimized empirically, and therefore can be slightly protracted.

While specific fragments of AR are lacking, the generation of full length CHD8 and AR in a baculovirus expression system has allowed the test of a direct interaction between CHD8 and AR. This experiment is one of the main goals of Aim 1 (Task 1b). SF9 cells were infected with baculovirus expressing 6-His tagged AR in the presence or absence of baculovirus expressing Flag-tagged CHD8. Lysates were prepared from these infected cells and samples were then immunoprecipitated with anti-flag antibodies. As shown in Annual Report 2008 Figure 1, only when cells are coinfected with Flag-tagged CHD8 can AR be immunoprecipitated with anti-flag antibodies. This result demonstrates a direct interaction between CHD8 and AR, and suggests that this interaction would not require β-catenin. As preliminary studies demonstrated a direct interaction between CHD8 and β-catenin, it is possible, however, to test for the creation of a tripartite complex between AR, CHD8 and β-catenin. In addition, once the GST-fragments of AR have been optimized and purified, it will be possible to test which domain of AR directly interacts with CHD8.

The second goal of Aim 1 was to extend the *in vitro* interaction studies to the description of the interaction between CHD8, AR, and β-catenin *in vivo* on chromatin using chromatin immunoprecipitation experiments (ChIPs). These experiments would benefit from the creation of stable β-catenin knock-down cells (Task1c). Constructs for the creation of these cell lines have been obtained from our consultant Dr. D. Turner. Several siRNA cassettes have been designed and tested. In order to quickly screen for constructs that effectively knockdown β-catenin activity, the following strategy was chosen: A colorectal cell line that has an activated Wnt signaling pathway (HCT116) was transfected with a reporter construct containing Tcf binding sites upstream of a luciferase reporter (TOP-Flash). In this cell line, robust reporter activity can be detected. These cells were also cotransfected with control siRNA constructs, or siRNA constructs targeting β-catenin. Of the transfected constructs, several displayed an appreciable knockdown of reporter activity, and the best construct was chosen for future experiments. Annual Report 2008 Figure 2 shows the effect of this construct on reporter activity. For comparison, cells were also transfected with a mutant form of Tcf4 that is incapable of binding β-catenin, and therefore acts as a dominant negative to inhibit the function of β-catenin. This data demonstrates that the constructed β-catenin siRNA construct effectively inhibits β-catenin mediated transcription, and that this inhibition is similar to levels produced when β-catenin can no longer interact with Tcf4 at the promoter. The β-catenin siRNA construct has been used in numerous attempts to create a stable LNCaP cell line. However, lines stabling expressing this construct have not been isolated. As outlined in the Contingencies section of Aim 1, an approach based on sorting transfected cells was then considered. siRNA vectors have been created that co-express the desired siRNA and also express a portion of the

IL2 receptor (IL2Rα subunit). Transfected cells can then be separated using magnetic bead technology (anti-IL2Rα monoclonal antibody, Dynal Biotech). This approach now circumvents the requirement of created stable knockdown cells. The results of Task 1c have now suggested Task 2a should be accomplished using this same technology, and vectors required for this task are currently under production.

Difficulties in creating stable cell lines have delayed the accomplishment of the second goal of Aim 1, and therefore Task 1d is still currently underway. Difficulties have also been encountered performing ChIP experiments with anti-β-catenin antibodies. Several different β-catenin antibodies have been tested for suitability in ChIP experiments, and these antibodies have failed to appreciable immunoprecipitate chromatin bound to β-catenin. Two recent studies have reported the identification by ChIP of β-catenin bound to the AR responsive PSA reporter (7,8). These studies used alternative antibodies that will be purchased and tested for functionality.

Year 2. The majority of the work proposed in months 12-24 addresses Specific Aim 2: Are CHD8 and β-catenin both required to affect AR transcriptional activity? As outlined in the 2008 Annual Report, several obstacles were encountered that required alternative approaches or additional effort. Therefore, this report outlines progress in several Tasks from Year 1 that were required for the continuation of Tasks planned for Year 2. Upon completion of these Tasks, progress was made on the majority of Tasks planned for Year 2. These advances have been sufficient to allow for the start of Tasks planned for Year 3.

Investigations listed in the 2008 Annual Report demonstrated a direct interaction between CHD8 and AR *in vitro* (Task 1a-b). Further proposed studies will then address the *in vivo* association of these proteins at endogenous promoters (Task 1d). Prior to completing this Task, a verification of the association of CHD8 and AR was performed *in vivo*. For this experiment three cell lines were used, LNCaP, 22RV1 and PC-3. LNCaP cells are an androgen dependent cell line and will be used in Tasks 1c-2c. 22RV1 and PC-3 cells are androgen independent cell lines and were included in this study for comparison. Nuclear extracts were prepared and samples were then immunoprecipitated with antibodies directed against CHD8. After extensive washing, immunoprecipitated samples were subjected to SDS-PAGE followed by Western blot analysis with antibodies against CHD8 or AR (Annual Report 2009 Figure 1). In agreement with the *in vitro* interaction data, CHD8 interacts with AR in the androgen dependent cell line, LNCaP. This result confirms their association and demonstrates that this cell line is a suitable choice for the completion of Aims 1 and 2. It was interesting to also note that CHD8 only interacted with AR in androgen dependent cell lines, although AR was present in all cell lines tested (Annual Report 2009 Figure 1). This result further strengthens the argument for Experiment 3.1 that addresses the requirement for CHD8 in progression from androgen dependent to androgen independent growth.

Experiments outlined in all three Tasks require the creation of stable cell lines that express shRNA to CHD8 and β-catenin (Task 1c, 2a, 3a). Various difficulties were encountered in Year 1, and these difficulties have been overcome allowing the creation of LNCaP cell lines with shRNA targeting CHD8 (Annual Report 2009 Figure 2). Importantly, the reduction of CHD8 is

independent of the presence of the AR ligand dihydrotestosterone (DHT). This cell line can now be used for the remainder of the Tasks outlined in 1 and 2. These cells also provide a viable alternative to the use of VCaP cells outlined in Task 3 should difficulties be encountered creating that cell line.

The creation of the LNCaP stable cell lines in Annual Report 2009 Figure 2 has allowed for the start of the experiments outlined in Task 2b to test the requirement of CHD8 in AR mediated transcription. Two endogenous androgen responsive targets were chosen for analysis, PSA and TMPRSS2. Analysis of the PSA gene was outlined in the original proposal, and TMPRSS2 is a newly identified androgen responsive gene that is fused to the ETS transcription factor in most prostrate cancers (9,10). The use of TMPRSS2 will further strengthen the results of these studies. LNCaP cells with shRNA to CHD8 or a control shRNA were treated with DHT or vehicle. cDNA was then isolated and analyzed by qPCR with primers to PSA or TMPRSS2 (Annual Report 2009 Figure 3). Consistent with the original hypothesis, upon induction with DHT depletion of CHD8 results in an approximate 2 fold decrease in the expression of both PSA and TMPRSS2. Of interest is that depletion of CHD8 results in an up regulation of these targets in the absence of DHT, and this up regulation is comparable to level of expression without CHD8 in the presence of DHT. These results suggest that these genes have now lost their ability to be regulated by AR. This is not due to the loss of AR expression as western blot analysis shows no change in AR protein levels upon depletion of CHD8 (data not shown).

The direct interaction of CHD8 with AR both *in vitro* and *in vivo*, and the fact that CHD8 is required for the DHT stimulated transcription of androgen target genes, further supports the hypothesis that CHD8 and AR interact at target genes to regulate androgen dependent transcription. The first step in testing this hypothesis is to demonstrate that CHD8 and AR can both bind to androgen responsive elements (AREs) at target genes. To test this, chromatin immunoprecipitation (ChIP) experiments were performed (Task1d). These studies were performed on AREs from both the PSA and TMPRSS2 genes in LNCaP cells treated with DHT. Annual Report 2009 Figure 4 demonstrates that CHD8 and AR are both bound to these promoters and the availability of the LNCaP stable cell lines in Annual Report 2009 Figure 2 will allow for the continuation of this Task (Task1d). In addition, two histone methylation marks were also tested for analysis in Task 2c.

Year 3.

The majority of the work proposed in months 24-36 addresses Specific Aim 3: Analyze the role of CHD8 in prostate cancer progression using xenograft models As outlined in the 2008 and 2009 Annual Reports, several obstacles were encountered that required alternative approaches or additional effort. Therefore, this report outlines progress in several Tasks from Years 1 and 2 that were required for the continuation of Tasks planned for Year 3. In addition, considerable effort was placed on finalizing experiments required for a publication on regulation of androgen-responsive transcription by CHD8. Data from these studies have also been accepted for publication in Molecular Endocrinology (see APPENDICES). The final date of publication will be May 2010.

Experiments outlined in the 2009 Annual Report demonstrate that CHD8 and AR are both bound to the PSA and TMPRSS2 genes in LNCaP cells treated with DHT. This finding allowed for further experiments outlined in Task 1d aimed at testing binding of CHD8 and AR to AREs of target genes. These experiments include addressing the recruitment of CHD8 under inducing and uninducing conditions. LNCaP cells were either mock-treated with ethanol or induced with DHT. ChIP experiments were then performed using antibodies against AR, CHD8 or normal rabbit IgG as a control. The immunoprecipitated chromatin was analyzed by quantitative PCR using primers directed against the TMPRSS2 ARE. Upon DHT induction, both AR and CHD8 bound the TMPRSS2 ARE region and not to a control promoter region located 7 kb upstream from the start site (Appendix A, Fig. 2A). As expected, AR was not bound to the target ARE region or the control region without induction by DHT. It was observed that CHD8 localized to the TMPRSS2 ARE region both with and without induction by DHT, indicating that CHD8 may be targeted to the TMPRSS2 ARE in a DHT-independent manner. Similar localization of CHD8 and AR was also observed on the AREs of the Prostate-Specific Antigen (PSA) promoter (Appendix A, Fig. S2).

While these results demonstrate that both CHD8 and AR can bind to the TMPRSS2 ARE in vivo, they do not address whether CHD8 and AR simultaneously occupy this ARE. Although this question was not originally proposed, addressing this question would provide critical information for the experiments proposed in Tasks 1d and 2c. To test simultaneous occupancy, re-ChIP assays were performed in the LNCaP cell line with and without DHT induction. Following cross-linking of the chromatin-protein complexes, a first immunoprecipitation utilized either CHD8 antibodies or normal rabbit IgG. Upon elution of the bound material, a second immunoprecipitation utilized either AR antibodies or normal rabbit IgG. Immunoprecipitated chromatin was then analyzed by quantitative PCR using primers directed against the TMPRSS2 ARE. As illustrated in Appendix A, Figure 2B, AR antibodies precipitated the TMPRSS2 ARE only after prior immunoprecipitation with CHD8 antibodies and only with DHT treatment. This result demonstrates that AR and CHD8 are simultaneously bound to the TMPRSS2 ARE upon DHT induction; strongly supporting the direct involvement of CHD8 in AR mediated transcription.

Experiments outlined in the 2009 Annual Report demonstrate that upon induction with DHT depletion of CHD8 results in a decrease in the expression of both PSA and TMPRSS2 genes. To further validate these findings, we expanded Task 2b to address the role of CHD8 in androgen mediated transcription in androgen independent cell lines. Again these experiments also included analysis of the TMPRSS2 and the PSA gene to further strengthen these studies. Experiments were performed in the AR-positive, androgen-independent line 22RV1 and also in the AR-negative, androgen-independent PC-3 and DU-145 cell lines. As expected in all three of these cell lines, DHT stimulation of TMPRSS2 gene expression was not observed (Appendix A, Fig. 4). Furthermore upon depletion of CHD8, no significant changes were observed in the pattern of TMPRSS2 or PSA gene expression. Similar results were obtained with PSA expression levels in each of these cell lines (Appendix A, Fig. S3). Taken together with our previous observation that CHD8 regulates AR-mediated expression in LNCaP cells, these results support the hypothesis that CHD8 plays an important role in androgen-responsive transcriptional activation.

Task 2c was designed to analyze the requirement of CHD8 in recruitment of AR coactivators to AR target genes. Following CHD8 depletion, cells were treated with DHT. ChIP experiments were then performed using antibodies against AR, CHD8, and normal rabbit IgG as a control. Analysis of the immunoprecipitated chromatin by quantitative PCR utilized primers directed against the ARE of TMPRSS2 (Appendix A, Fig.5). As expected, depletion of CHD8 results in a significant reduction of CHD8 bound to the TMPRSS2 ARE. In the presence of CHD8, AR was appropriately recruited to the TMPRSS2 ARE. However, upon depletion of CHD8, the binding of AR to the ARE of TMPRSS2 was severely abrogated. This result indicates that CHD8 is required for the proper binding of AR to target sites in the TMPRSS2 gene, and therefore CHD8 is acting upstream of AR binding. As AR binding is required to recruit AR coactivators, the role of CHD8 in coactivator binding will not be addressed further.

Experiments in all three Aims required depletion of CHD8 in VCaP cells. Various difficulties depleting CHD8 in these cells were encountered in Year 1, and these difficulties have been overcome by using the LNCaP cell line. Experiments outlined in Aim 3 required VCaP cells for the creation of prostate cancer xenograft models in nude mice. As LNCaP cells are not ideal for the creation of xenograft models (D. Robins, personal communication), initial experiments were designed to test if CHD8 plays an important role in androgen-dependent cell growth of LNCaP cells. CHD8 depleted or wild type LNCaP cells induced with 4 nM of the synthetic androgen R1881 or mock-treated with ethanol. While R1881 treatment of the control LNCaP cells results in a marked increase in proliferation, R1881-induced proliferation is strongly inhibited by CHD8 depletion (Appendix A, Fig. 6). This result shows that CHD8 is indeed required for androgen-induced cell proliferation of LNCaP cells. More importantly, this result demonstrates that CHD8 may be novel therapeutic target for prostate cancer.

An alternative in Task 3c would utilize mouse prostate tissues obtained from the laboratory of Dr. Robins. Her laboratory has recently developed a system to look at differential initiation and progression of prostate tumors(11). In addition, VCaP xenograft samples are also available. Tissue microarray samples for CHD8 immunohistochemistry (normal prostate vs. TRAMP tumor, intact prostate vs. castrate prostate, and VCaP xenografts) were obtained. Once CHD8 expression is verified in these tissues, samples will be isolated and analyzed by ChIP for changes in chromatin structure and CHD8 recruitment. These experiments are currently ongoing.

KEY RESEARCH ACCOMPLISHMENTS

- **Year 1**
- Recombinant proteins have been produced for the study of the interactions between CHD8, AR, and β-catenin (Task 1a). Production of several additional fragments is currently underway.
- Protein interaction studies have been performed to demonstrate the direct interaction of CHD8 and AR (Task 1b). This result will now direct research towards the investigation of a tripartite CHD8/AR/β-catenin complex.
- siRNA constructs have been made for the *in vivo* knockdown of β-catenin in stable cell lines.

- Creation of the above cell lines was not successful (Task 1c), and therefore, as outlined in the Contingency section, a system for sorting transfected cells has now been developed. These will be used in Task 1d and Task 2a.
- Chromatin immunoprecipitation experiments were commenced (Task 1d). However, two difficulties were encountered. These are currently being resolved using the above technique of cell sorting, and also by the purchase of β-catenin antibodies suitable for chromatin immunoprecipitation.

- **Year 2**
- Protein interaction studies have been performed to demonstrate the interaction of CHD8 and AR *in vivo*. This result is a continuation of Task 1b.
- Stable LNCaP cell lines have been made for the *in vivo* knockdown of CHD8 (Task 1c and Task 2a).
- Chromatin immunoprecipitation experiments were commenced to detect the association of AR and CHD8 at androgen responsive promoters (Task 1d). These assays also included various antibodies directed against histone methylation marks (Task 2c)
- A direct role for CHD8 in AR mediated transcription has been demonstrated using CHD8 knockdown LNCaP cells (Task 2b).
- Although a stable VCaP cell line has yet to be created (Task 3a), the creation of a stable knockdown of CHD8 in LNCaP provides a workable alternative for the experiments outlined in Year 3 (Task 3b and 3c).

- **Year 3**
- Chromatin immunoprecipitation experiments were continued to detect the association of AR and CHD8 at androgen responsive promoters (Task 1d). These results demonstrate that unlike AR, CHD8 is recruited to androgen responsive promoters in an androgen independent manner.
- ReChIP experiments demonstrate that CHD8 and AR simultaneously bind to the TMPRSS2 ARE upon DHT treatment (Task 1d).
- A direct role for CHD8 in AR mediated transcription has been demonstrated in androgen dependent cell lines, but not in androgen independent cell lines (Task 2b).
- CHD8 is required for the proper binding of AR to target sites in the TMPRSS2 ARE and therefore CHD8 is acting upstream of AR binding (Task 2b).
- CHD8 is required for androgen-induced cell proliferation of LNCaP cells and therefore CHD8 is novel therapeutic target for prostate cancer cell growth (Task 3a).
- Tissue microarray samples for CHD8 immunohistochemistry were obtained to test for suitability in Task 3c.

REPORTABLE OUTCOMES (All Years)

The data presented here has been presented locally at the University of Michigan, both in the department, as well as interdepartmentally. This data has also been presented for an invited seminar at the University of Toledo, Department of Pharmacology. In addition, portions of these data have also been presented at the ASBMB Transcriptional Regulation by Chromatin and RNA Polymerase II meeting in Lake Tahoe, CA, and also at the Michigan State University

Summer Symposium on Transcriptional Regulation and Systems Biology meeting in East Lansing, MI. This data will also be presented at the Experimental Biology – ASBMB Annual Meeting 2010 this April in Anaheim CA.

Data from these studies have also been accepted for publication in *Molecular Endocrinology* (see APPENDICES). The final date of publication will be May 2010.

PUBLICATIONS

Menon T, Yates JA and Bochar DA. 2010. Regulation of Androgen-Responsive Transcription by the Chromatin Remodeling Factor CHD8. Mol Endocrinol. EPub ahead of print doi:10.1210/me.2009-0421 (Attached)

MEETING ABSTRACTS

Bochar, D. A, Thompson, B. A., Menon, T., and J. A. Yates. CHD8 is an ATP-dependent Remodeling Factor that Regulates β-Catenin Target Genes. MSU Summer Symposium on Transcriptional Regulation and Systems Biology. July 18 – July 20, 2008.

Bochar, D. A, Thompson, B. A., Menon, T., and J. A. Yates. A functional role for CHD8 in transcriptional regulation through the modulation of chromatin structure. ASBMB Transcriptional Regulation by Chromatin and RNA Polymerase II Meeting, October 16 – October 20, 2008.

Bochar, D. A, Menon, T., and J. A. Yates A functional role for CHD8 in transcriptional regulation through the modulation of chromatin structure. Experimental Biology – ASBMB 2010, April 24th – April 28th 2010.

PERSONNEL RECEVING SUPPORT

Daniel A. Bochar (PI), Assistant Professor of Biological Chemistry.

Diane M. Robins (Co-PI), Professor of Human Genetics, Research Scientist in Reproductive Sciences.

Joel A Yates, Graduate Student in Chemical Biology.

CONCLUSION

The majority of the experiments proposed in this grant have been completed. We have demonstrated a direct interaction both in vivo and in vitro between CHD8 and AR. We have shown that CHD8 is localized to the TMPRSS2 and PSA AREs. We have also shown that CHD8 is localized simultaneously with AR to the TMPRSS2 ARE. Surprisingly, CHD8 is different from many other AR coactivators in that it is localized to the TMPRSS2 and PSA AREs prior to androgen induction. We have demonstrated that CHD8 is required for optimal DHT induced transcriptional activation of the TMPRSS2 and PSA genes in the androgen dependent LNCaP cell line, but not in several other androgen independent lines. Further experiments

suggest a mechanism for the role of CHD8 in enhancing or stabilizing the interaction of AR with chromatinized targets as depletion of CHD8 results in loss of AR bound to the TMPRSS2 ARE. Lastly, the physiological importance of CHD8 in the regulation of R1881-induced cell proliferation is also demonstrated, as androgen-induced proliferation of LNCaP cells is strongly inhibited by CHD8 depletion. Our results further strengthen our hypothesis that CHD8 is a novel diagnostic, preventative, or therapeutic target in prostate cancer.

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APPENDICES

A. Menon T, Yates JA and Bochar DA. 2010. Regulation of Androgen-Responsive Transcription by the Chromatin Remodeling Factor CHD8. Mol Endocrinol. EPub ahead of print doi:10.1210/me.2009-0421

Molecular Endocrinology. First published ahead of print March 22, 2010 as doi:10.1210/me.2009-0421

Regulation of Androgen-Responsive Transcription by the Chromatin Remodeling Factor CHD8

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The androgen receptor (AR) mediates the effect of androgens through its transcriptional function during both normal prostate development and in the emergence and progression of prostate cancer. AR is known to assemble coactivator complexes at target promoters to facilitate transcriptional activation in response to androgens. Here we identify the ATP-dependent chromatin remodeling factor chromodomain helicase DNA-binding protein 8 (CHD8) as a novel coregulator of androgen-responsive transcription. We demonstrate that CHD8 directly associates with AR and that CHD8 and AR simultaneously localize to the TMPRSS2 enhancer after androgen treatment. In the LNCaP cell line, reduction of CHD8 levels by small interfering RNA treatment severely diminishes androgen-dependent activation of the TMPRSS2 gene. We demonstrate that the recruitment of AR to the TMPRSS2 promoter in response to androgen treatment requires CHD8. Finally, CHD8 facilitates androgen-stimulated proliferation of LNCaP cells, emphasizing the physiological importance of CHD8. Taken together, we present evidence of a functional role for CHD8 in AR-mediated transcriptional regulation of target genes. **(*Molecular Endocrinology* 24: 0000–0000, 2010)**

Prostate cancer is currently the most commonly diagnosed form of cancer and the second leading cause of cancer deaths among males in the United States (1). Androgens, such as testosterone and dihydrotestosterone (DHT), regulate transcription via the androgen receptor (AR) and play a critical role in the normal growth and function of the prostate (2). The dysregulation of AR signaling has also been implicated in the development and progression of prostate cancer. An example includes the aberrant regulation of the ETS family of transcription factors, which are the most frequently overexpressed protooncogenes in prostate cancer (3). Overexpression commonly results from gene fusions between the 5′ untranslated region of the androgen-responsive TMPRSS2 gene to members of the ETS family, placing these protooncogenes under the control of TMPRSS2 androgen-responsive elements (AREs) (4).

Over 80% of prostate cancers are androgen dependent at initial diagnosis, and thus most common therapeutic approaches are directed toward androgen ablation or inhibition of AR (5). Initially, these methods prove to be effective in causing the regression of androgen-dependent tumors, thus highlighting the role of AR activity in early prostate tumorigenesis. However, these treatments often ultimately fail due to progression of the prostate cancer to a hormone-refractory state (6). Some of the proposed mechanisms for this transition to an androgen-independent state include the increased expression of AR or its associated factors, mutations of AR that make it responsive to a broader spectrum of ligands, activation of the receptor through alternate pathways, and the altered function of AR coregulators (7–9). Studying the association and interplay of AR with its many coregulators is therefore critical for the development of novel and more effective therapies for prostate cancer.

Several AR coactivators have been implicated in prostate cancer, specifically in AR-mediated control of primary prostate cancer tumorigenesis and progression (10). Alterations in levels or function of AR coactivators have also been proposed to contribute to the emergence of the

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Abbreviations: AR, Androgen receptor; ARE, androgen-responsive element; CHD, chromodomain helicase DNA-binding; ChIP, chromatin immunoprecipitation; DHT, dihydrotestosterone; FBS, fetal bovine serum; NP-40, Nonidet P-40; PPAR, peroxisome proliferator-activated receptor; PSA, prostate-specific antigen; SDS, sodium dodecyl sulfate; siRNA, small interfering RNA.

hormone-refractory disease (11). A recurring function for AR coactivators is the catalysis of site-specific modification events, such as histone acetylation or methylation, at target promoters to modify the chromatin structure (12, 13). These events are not just limited to covalent modifications of chromatin; ATP-dependent chromatin remodeling enzymes also play a key role in AR-mediated transcriptional activation (14–22).

ATP-dependent chromatin remodeling enzymes use energy derived from ATP hydrolysis to mobilize and modulate nucleosomes (23). A characteristic feature of these enzymes is the presence of a central ATPase component referred to as the Snf2 helicase domain. The Snf2 superfamily is then classified into families based upon the presence of other conserved domains (24). One such family is the chromodomain helicase DNA-binding (CHD) family of enzymes. In mammals, this family can be further divided into three subfamilies: CHD1-CHD2, CHD3-CHD5, and CHD6-CHD9 (25). Although the CHD1-2 and CHD3-5 subfamily proteins have been well studied in the context of their chromatin remodeling activity and their functional role in transcriptional regulation, relatively little is known about the CHD6–9 subfamily.

Previous work from our laboratory has shown that CHD8 is an ATP-dependent chromatin remodeling enzyme involved in transcriptional regulation of β-catenin-responsive genes as well as at the Hox locus (26, 52). Other studies have linked CHD8 to CTCF-mediated insulator function (27), to RNA polymerase III transcription in association with hStaf (28), to control of p53-mediated apoptosis (29), and to RNA polymerase II-associated transcription of the cyclin E2 gene (30). Thus, CHD8 exhibits a diverse range of functions in transcriptional regulation. A recent report shows that CHD8 is required for optimal estrogen-responsive induction of cyclin E2 (31), raising the possibility that CHD8 may also be involved in transcriptional regulation by other nuclear receptors.

Here we report that in the androgen-dependent prostate cancer cell line LNCaP, endogenous CHD8 associates with AR as determined by coimmunoprecipitations from nuclear extract. We demonstrate that this association is due to a direct physical interaction by coexpressing recombinant proteins in insect cells followed by coimmunoprecipitation experiments. Using chromatin immunoprecipitation (ChIP) experiments, we demonstrate that CHD8 is present at the TMPRSS2 ARE both before and after induction with DHT. Using re-ChIP experiments, we show AR and CHD8 simultaneously localize to the TMPRSS2 ARE after DHT treatment. In androgen-dependent LNCaP cells, reduction of CHD8 levels by small interfering RNA (siRNA) treatment severely diminishes DHT-dependent activation of the TMPRSS2 gene. However, in several androgen-independent cells, reduction of CHD8 did not alter the expression of the TMPRSS2 gene. Thus, CHD8 is required for optimal AR-mediated transcriptional activation of the TMPRSS2 gene in an androgen-dependent context. To investigate the function of CHD8, we performed ChIP experiments for AR under conditions of CHD8 depletion. Using this approach, we found that the recruitment of AR to the TMPRSS2 promoter in response to DHT treatment requires the presence of CHD8. Finally, by using siRNA against CHD8, we demonstrate that CHD8 facilitates proliferation of LNCaP cells in response to treatment with the synthetic androgen R1881. Taken together, we present evidence of a functional role for CHD8 in AR-mediated transcriptional activation of target genes.

Results

Identification of CHD8 as a novel AR interacting protein

To identify novel chromatin-remodeling enzymes that may function as cofactors for AR in the progression of prostate cancer, publicly available gene expression sets were examined using the ONCOMINE database (www.oncomine.org; search gene CHD8) (32). This investigation revealed that CHD8 was found to be up-regulated in several prostate cancer *vs.* normal tissue data sets (33–37). We have previously identified and characterized CHD8 as an ATP-dependent chromatin remodeling enzyme involved in the regulation of gene transcription (26). Given the fact that many AR coactivators are up-regulated in prostate cancer (22), the finding that CHD8 is up-regulated in several studies suggests that CHD8 may function in the regulation of AR-mediated transcription.

AR coregulators commonly function through a direct interaction between the receptor and the coregulator protein. We therefore examined whether there is a physical association between CHD8 and AR. To test this possibility, coimmunoprecipitations of endogenous proteins were performed from several different prostate cancer cell lines. Nuclear extracts were prepared from the androgen-dependent LNCaP cell line as well as from the androgen-independent cell lines 22RV1, PC-3, and DU-145 (38–42). AR was detected in the LNCaP and 22RV1 nuclear extracts but not in extracts from PC-3 or DU-145 (Supplemental Fig. 1 published on The Endocrine Society's Journals Online web site at http://mend.endojournals.org). Immunoprecipitations were then performed from the AR-positive extracts using anti-CHD8 polyclonal antibodies. After Western blot analysis with antibodies for CHD8 and AR, it was observed that CHD8 antibodies

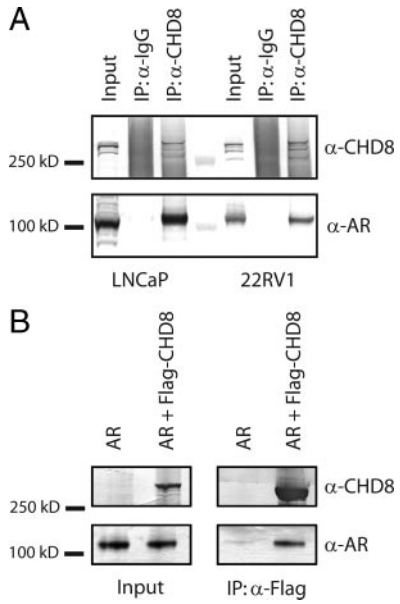

FIG. 1. CHD8 interacts with AR. A, Nuclear extracts were prepared from the indicated cell lines and immunoprecipitated with CHD8 antibodies. After washing, the input and immunoprecipitated samples (IP) were subjected to Western blot analysis using the indicated antibodies. B, Cellular extracts were prepared from SF9 cells after coinfection with the indicated viruses. Immunoprecipitations were performed with anti-Flag antibody-linked M2 agarose beads. Immunoprecipitated samples were subjected to Western blot analysis using the indicated antibodies.

immunoprecipitate both CHD8 and AR, whereas normal rabbit IgG fails to precipitate any detectable AR or CHD8 (Fig. 1A). These results demonstrate that endogenous AR and CHD8 do indeed interact in these cell lines.

To further investigate the interaction between AR and CHD8, coimmunoprecipitation experiments were performed with recombinant proteins produced in a baculovirus expression system. SF9 cells were infected with either baculovirus encoding AR or coinfected with AR and Flag-CHD8-encoding baculoviruses. After infection, cell extracts were prepared and immunoprecipitation experiments were performed using anti-Flag antibodies. As shown in Fig. 1B, AR was immunoprecipitated with anti-Flag antibodies only in the presence of Flag-CHD8. This result confirms the previously identified interaction of CHD8 and AR and also demonstrates that this association is due to a direct physical interaction between these two proteins.

CHD8 and AR simultaneously bind the TMPRSS2 ARE

TMPRSS2 is a prostate-specific transmembrane serine protease expressed under the control of the AR. The TMPRSS2 locus contains a previously defined enhancer region located approximately 13.5 kb upstream from the start site that contains an ARE that is required for the androgen-responsive expression of this gene (43). In prostate cancer, overexpression of members of the ETS family of protooncogenes commonly results from gene fusions between the 5′ untranslated region of TMPRSS2 to members of the ETS family, placing these protooncogenes under the control of TMPRSS2 AREs (4).

The direct physical interaction between AR and CHD8 suggests that CHD8 may be recruited to endogenous AR target promoters, such as TMPRSS2, to modulate chromatin structure. To examine this hypothesis, ChIP experiments were performed in LNCaP cells because AR is known to regulate androgen-responsive gene expression in these cells. LNCaP cells were either mock treated with ethanol or induced with DHT for 6 h. ChIP experiments were then performed using antibodies against AR, CHD8, or normal rabbit IgG as a control. The immunoprecipitated chromatin was analyzed by quantitative PCR using primers directed against the TMPRSS2 ARE. Upon DHT induction, both AR and CHD8 bound the TMPRSS2 ARE region and not to a control promoter region located 7 kb upstream from the start site (Fig. 2A). As expected, AR was not bound to the target ARE region or the control

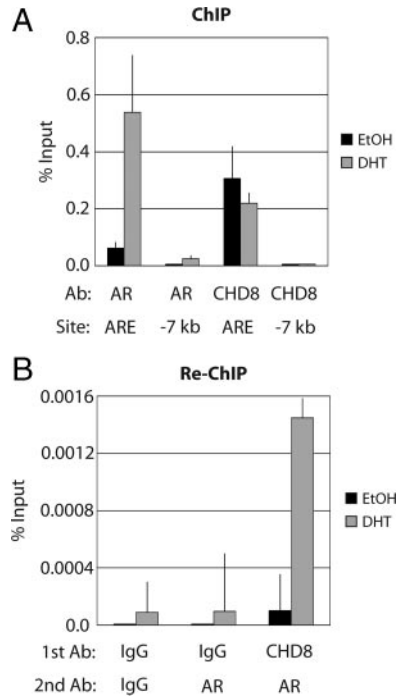

FIG. 2. CHD8 and AR colocalize to the TMPRSS2 ARE. A, LNCaP cells were treated with ethanol (EtOH) or 10 nM DHT for 6 h. Chromatin was cross-linked *in vivo* with formaldehyde. Cells were lysed, and ChIP was performed with the indicated antibodies (Ab). Bound DNA was detected by quantitative PCR using primers to the ARE of TMPRSS2 (ARE) or a control TMPRSS2 promoter region (−7 kb). Control IgG-precipitated samples were less than 0.005% of input and therefore are not shown. Data are representative of multiple experiments. B, LNCaP cells were treated as in A. Re-ChIP experiments were performed by successively immunoprecipitating the cross-linked chromatin with the indicated antibodies. Bound DNA was detected by quantitative PCR using primers to the TMPRSS2 ARE. Data are representative of multiple experiments.

region without induction by DHT. It was observed that CHD8 localized to the TMPRSS2 ARE region both with and without induction by DHT, indicating that CHD8 may be targeted to the TMPRSS2 ARE in a DHT-independent manner. Similar localization of CHD8 and AR was also observed on the AREs of the prostate-specific antigen (PSA) promoter (Supplemental Fig. 2).

Although these results demonstrate that both CHD8 and AR can bind to the TMPRSS2 ARE *in vivo*, they do not address whether CHD8 and AR simultaneously occupy this ARE. To test this hypothesis, re-ChIP assays were performed in the LNCaP cell line with and without DHT induction. After cross-linking of the chromatin-protein complexes, a first immunoprecipitation used either CHD8 antibodies or normal rabbit IgG. Upon elution of the bound material, a second immunoprecipitation used either AR antibodies or normal rabbit IgG. Immunoprecipitated chromatin was then analyzed by quantitative PCR using primers directed against the TMPRSS2 ARE. As illustrated in Fig. 2B, AR antibodies precipitated the TMPRSS2 ARE only after prior immunoprecipitation with CHD8 antibodies and only with DHT treatment. This result demonstrates that AR and CHD8 are indeed simultaneously bound to the TMPRSS2 ARE upon DHT induction.

CHD8 activates AR-dependent TMPRSS2 gene expression

Having established a direct physical interaction between AR and CHD8 and their simultaneous colocalization at the TMPRSS2 ARE, we next examined whether CHD8 has a functional role in the regulation of TMPRSS2 gene expression. This was accomplished by using a vector-based siRNA approach to deplete endogenous CHD8 (44). LNCaP cells were transfected with either a control siRNA construct or with an siRNA construct directed against CHD8. These vectors also coexpress a puromycin resistance marker and therefore allow for the selection of transfected cells. After selection with puromycin for 48 h, the cells were treated with either ethanol or DHT for 6 h, and cDNA was prepared for quantitative PCR analysis of TMPRSS2 expression levels. As expected, in the cells treated with control siRNA, TMPRSS2 expression was induced approximately 6-fold upon DHT treatment (Fig. 3A). In contrast, under conditions of CHD8 depletion, DHT-induced expression of TMPRSS2 was almost completely abrogated (Fig. 3A). CHD8 siRNA treatment resulted in lowered PSA expression in both uninduced and DHT-induced cells. However, PSA expression may still be responsive to hormonal induction (Fig. 3B). The effectiveness of siRNA depletion is shown in Fig. 3C, where we see considerable reduction of

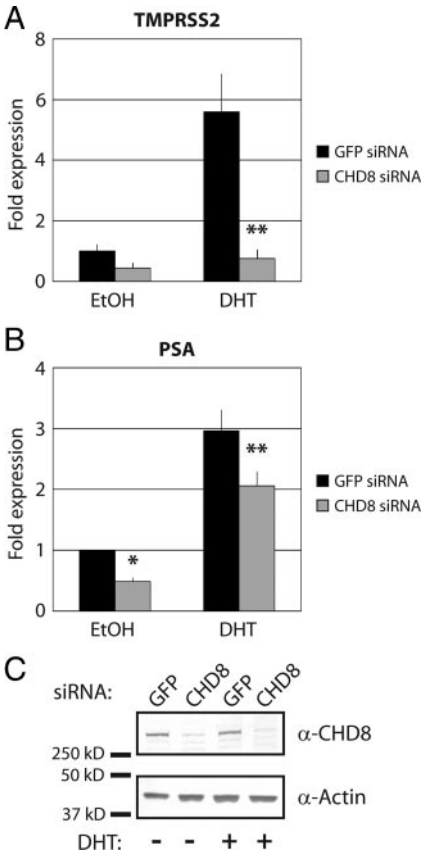

FIG. 3. CHD8 activates AR-mediated transcription of the TMPRSS2 gene. LNCaP cells were transfected with the indicated siRNA constructs. After selection of the transfected cells, cultures were treated with ethanol (EtOH) or 10 nM DHT for 6 h. A and B, Total RNA was isolated, and TMPRSS2 expression (A) or PSA expression (B) was analyzed by quantitative RT-PCR. *, $P < 0.05$; **, $P < 0.01$ by Student's *t* test. C, Efficiency of CHD8 knockdown was determined by Western blot analysis with CHD8 antibodies. Actin is blotted as a loading control.

CHD8 levels by Western blot analysis. This result demonstrates that CHD8 is required for optimal androgen-induced transcriptional activation of the AR target gene TMPRSS2.

As shown in Fig. 1A, we observed an *in vivo* interaction between CHD8 and AR not only in the androgen-responsive LNCaP cell line but also in the androgen-independent 22RV1 cell line. This led us to question whether regulation of TMPRSS2 expression by CHD8 is dependent on direct AR interaction and/or in response to androgen stimulation. We tested CHD8 regulation of TMPRSS2 expression in the AR-positive, androgen-independent line 22RV1. We also tested CHD8 regulation of TMPRSS2 expression in the AR-negative, androgen-independent PC-3 and DU-145 cell lines. Expression of TMPRSS2 and PSA is not responsive to androgen treatment in any of these three cell lines either due to the absence of functional AR (PC-3 and DU-145) or due to transition into a hormone-refractory state (22RV1) (45–47). As expected in all three of these cell lines, DHT stimulation of

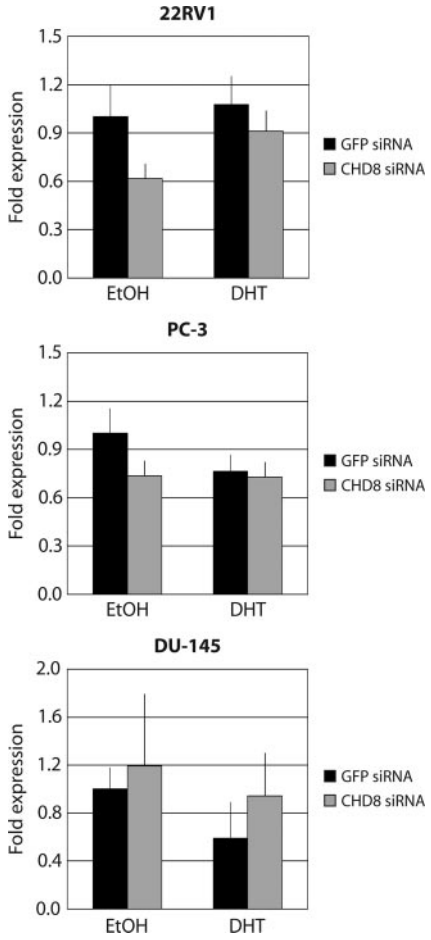

FIG. 4. CHD8 activates TMPRSS2 in an androgen-dependent context. The indicated androgen-independent cell lines were transfected with the specified siRNA constructs. After selection of the transfected cells, cultures were treated with ethanol (EtOH) or 10 nM DHT for 6 h. Total RNA was isolated, and TMPRSS2 expression was analyzed by quantitative RT-PCR.

TMPRSS2 gene expression was not observed (Fig. 4). Furthermore, upon depletion of CHD8, no significant changes were observed in the pattern of TMPRSS2 gene expression. Similar results were obtained with PSA expression levels in each of these cell lines (Supplemental Fig. 3). Taken together with our previous observation that CHD8 regulates AR-mediated expression in LNCaP cells, these results support the hypothesis that CHD8 plays an important role in androgen-responsive transcriptional activation.

CHD8 is required for optimal binding of AR to the TMPRSS2 ARE

A possible function for CHD8 in androgen-dependent TMPRSS2 transcriptional activation could be that CHD8 plays a role in modulating the chromatin structure to enhance AR binding to target sites. To test this hypothesis, we examined the effects of CHD8 depletion on the localization of AR at the TMPRSS2 ARE site. As outlined above, endogenous CHD8 was depleted via siRNA. After

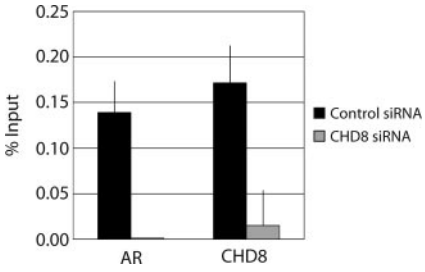

FIG. 5. CHD8 is required for optimal androgen-responsive binding of AR to the TMPRSS2 ARE. LNCaP cells were transfected with the indicated siRNA constructs. After selection of the transfected cells, cultures were treated with ethanol or 10 nM DHT for 6 h. Chromatin was cross-linked *in vivo* with formaldehyde. Cells were lysed, and ChIP was performed with the indicated antibodies. Bound DNA was detected by quantitative PCR using primers to the TMPRSS2 ARE. Control IgG-precipitated samples were less than 0.005% of input and therefore are not shown. Shown is a typical result from multiple experiments.

puromycin selection, cells were treated with DHT for 6 h. ChIP experiments were then performed using antibodies against AR, CHD8, and normal rabbit IgG as a control. Analysis of the immunoprecipitated chromatin by quantitative PCR used primers directed against the ARE of TMPRSS2 (Fig. 5). As expected, depletion of CHD8 results in a significant reduction of CHD8 bound to the TMPRSS2 ARE. In the presence of CHD8, AR was appropriately recruited to the TMPRSS2 ARE. However, upon depletion of CHD8, the binding of AR to the ARE of TMPRSS2 was severely abrogated. This result indicates that CHD8 is required for the proper binding of AR to target sites in the TMPRSS2 ARE. Because CHD8 is present at these target promoters even before DHT-induced AR recruitment, it is possible that the chromatin-remodeling activity of CHD8 may be required to allow AR to bind the TMPRSS2 ARE upon androgen induction.

CHD8 is required for androgen-induced cell proliferation

Our results indicate that CHD8 is required for the optimal transcriptional activation of the TMPRSS2 gene upon induction by DHT and that CHD8 is required for the appropriate androgen-responsive recruitment of AR to target promoters. Therefore, it is likely that CHD8 plays an important role in androgen-dependent cell growth of LNCaP cells. Proliferation assays were conducted in LNCaP cells transfected with either control or CHD8-targeting siRNA constructs. After selection with puromycin, cells were either induced with 4 nM of the synthetic androgen R1881 or mock treated with ethanol. Although R1881 treatment of the control LNCaP cells results in a marked increase in proliferation, R1881-induced proliferation is strongly inhibited by CHD8 depletion (Fig. 6). Thus, it appears that CHD8 is indeed re-

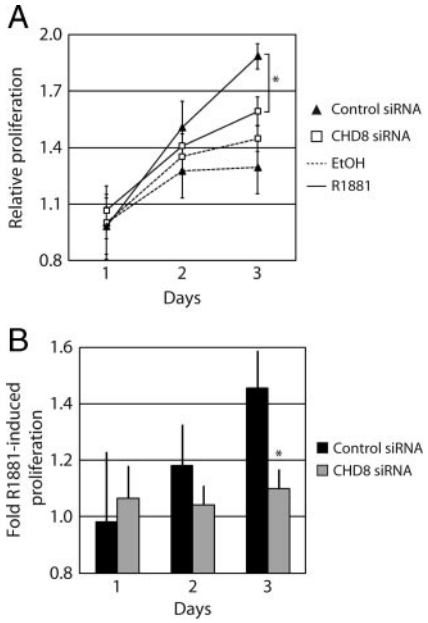

FIG. 6. CHD8 regulates androgen-dependent cell proliferation. LNCaP cells were transfected with the indicated siRNA constructs. After selection of the transfected cells, cultures were treated with ethanol (EtOH) or 4 nM R1881. At the indicated time points, proliferation was determined using a luminescent-based assay of metabolically active cells. A, Data are expressed as relative proliferation, which is calculated as the luminescent signal for each condition normalized to the uninduced sample at d 1. B, Data are expressed as fold R1881-induced proliferation, which is calculated as the ratio of the luminescent signals from the induced to the uninduced cells for each siRNA treatment at each indicated time point. *, $P < 0.02$ by Student's *t* test.

quired for androgen-induced cell proliferation of LNCaP cells.

Discussion

Analysis of publicly available microarray data sets comparing the expression profiles of normal prostate tissue to prostate tumors led to the discovery that CHD8 is up-regulated in prostate cancer. Because several AR coactivators also exhibit similar up-regulation in prostate cancer and many of these coactivators are known chromatin-remodeling enzymes (48), we hypothesized that CHD8 also functions as a coactivator for AR via the modulation of chromatin structure. In support of this hypothesis, we have demonstrated a direct interaction both *in vivo* and *in vitro* between CHD8 and AR. Furthermore, we have shown that CHD8 is localized simultaneously with AR to the TMPRSS2 ARE. However, CHD8 is unique from many other AR coactivators in that it is localized to the TMPRSS2 ARE before androgen stimulation. In conjunction with the localization of CHD8 to the TMPRSS2 ARE, we demonstrate that CHD8 is required for optimal DHT-induced transcriptional activation of the TMPRSS2 gene. Mechanistically, it appears CHD8 may be functioning in enhancing or stabilizing the interaction of AR with chromatinized targets because depletion of CHD8 results in loss of AR bound to the TMPRSS2 ARE. Finally, the physiological importance of CHD8 in the regulation of R1881-induced cell proliferation is also demonstrated, because androgen-induced proliferation of LNCaP cells is strongly inhibited by CHD8 depletion.

The role of CHD8 in nuclear hormone signaling is additionally supported by other lines of evidence. CHD8 is a member of the highly related CHD6-9 subfamily of proteins. Several members of this family have been shown to functionally associate with nuclear hormone receptors. CHD9 (CReMM/PRIC320) has been shown to interact with peroxisome proliferator-activated receptor (PPAR)-α, constitutive androstane receptor, estrogen receptor-α, retinoid X receptor, and glucocorticoid receptor and has also been shown to function as a coactivator for PPARα (49, 50). CHD7 has also been isolated as a component of a corepressor complex that inhibits PPARγ-mediated transcription (51). Recently, CHD8 has also been reported to be required for the estrogen-mediated up-regulation of the cyclin E2 gene (31). Taken together with our studies on CHD8 and AR, these results suggest that the CHD6-9 family is an important regulator of nuclear hormone signaling.

Deciphering the mechanistic role of CHD8 in transcriptional regulation is complicated by the numerous functions reported for CHD8. Previous work from our group has shown that CHD8 is an ATP-dependent chromatin-remodeling enzyme involved in transcriptional regulation of β-catenin-responsive genes (26). However, CHD8 was found to act in the negative regulation of activated β-catenin-responsive genes, unlike our current report of a role for CHD8 in the activation of TMPRSS2 in response to androgens. This suggests that CHD8 can differentially regulate numerous target genes. Indeed, expression profiling of control and CHD8-depleted cells identified transcripts both positively and negatively regulated by CHD8 (30).

Further insight into the function of CHD8 can be found by examining the reported functions of Kismet, the *Drosophila* ortholog of CHD8. Kismet was originally identified as an extragenic suppressor of Polycomb and therefore assigned as a member of the trxG of activators (53). Further studies revealed that Kismet assists in an early step in transcriptional elongation (54, 55). These reported data are consistent with CHD8 regulating the cyclin E2 gene via interactions with the elongating polymerase (30). AR plays a role in not only transcriptional initiation but also transcriptional elongation. AR has been reported to interact with COBRA1 (NELF-B), a subunit of negative elongation factor (NELF), and depletion

of endogenous NELF-B enhances DHT-mediated transcriptional activation (56). AR also interacts with the positive elongation factor P-TEFb, and this interaction serves to enhance transcriptional elongation (57). In addition, AR has been shown to regulate transcriptional initiation as well as elongation via interactions with the general transcription factors TFIIF and TFIIH (58, 59). Taken together with our current studies, these reports suggest CHD8 could possibly be regulating AR-mediated transcription by modulating transcriptional elongation.

In this study, we have identified the binding of CHD8 to the TMPRSS2 enhancer region located approximately 13.5 kb upstream from the start site. These data initially seem to be at odds with the model predicted above. However, the investigation of various nuclear receptors binding to DNA at both proximal and distal sites accompanied by reports of RNA polymerase II localization to these sites suggests that enhancer/promoter looping may play an important role in the regulation of nuclear hormone-regulated transcription (60). Indeed, a direct interaction is reported between the TMPRSS2 −13.5-kb enhancer and the promoter region (43). Therefore, the recruitment of CHD8 to the TMPRSS2 distal enhancer does not preclude CHD8 functioning in transcriptional elongation. More experiments need to be performed to determine the precise point of action for CHD8 in the transcriptional cycle.

In summary, the activity of AR is critical for normal prostate development and function but also plays a major role in the development and progression of prostate cancer. Understanding the mechanisms of transcriptional regulation by AR and AR-associated cofactors is critical to the development of new therapies for prostate cancer. Here we report the characterization of a novel AR-associated cofactor required for the proper regulation of the androgen-responsive gene TMPRSS2. Our results present CHD8 as a novel diagnostic, preventative, or therapeutic target in prostate cancer.

Materials and Methods

Cell culture

LNCaP, 22RV1, PC-3, and DU-145 cell lines were obtained from the American Type Culture Collection (Manassas, VA). Cell lines were maintained in RPMI medium 1640 (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS) (Hyclone, Logan, UT) and 1× penicillin-streptomycin-glutamine (Invitrogen) at 37 C in 5% CO_2. Twenty-four hours before androgen stimulation, cells were switched to phenol red-free RPMI medium 1640 (Invitrogen) supplemented with 10% dextran/charcoal-stripped FBS (Hyclone) and 1× penicillin-streptomycin-glutamine. DHT was dissolved in ethanol and used at a final concentration of 10 nM. R1881 was dissolved in ethanol and used at a final concentration of 4 nM. SF9 insect cells were cultured at 25 C in 1× Grace's insect medium (Invitrogen) containing 10% FBS and 1× penicillin-streptomycin-glutamine.

Antibodies

Rabbit polyclonal antibodies raised against CHD8 were previously described (26). Rabbit polyclonal (N-20) and mouse monoclonal AR antibodies for Western blotting were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and from BD Pharmingen (San Diego, CA), respectively. Mouse monoclonal (441) AR antibodies for ChIP were obtained from Santa Cruz Biotechnology. Normal rabbit IgG and actin control antibodies were purchased from Sigma Chemical Co. (St. Louis, MO). Antirabbit IgG conjugated to alkaline phosphatase was purchased from Promega (Madison, WI).

Recombinant protein production

Recombinant baculoviruses were used to express AR and CHD8 in SF9 insect cells. The AR baculovirus was a kind gift from J. T. Dalton (Ohio State University, Columbus, OH). The Flag-CHD8 baculovirus was previously described (26). For protein interaction studies, 5×10^6 SF9 cells were coinfected with 1 ml each of AR and Flag-CHD8 baculovirus and incubated at 25 C for 2 d. Cells were then harvested by centrifugation at $500 \times g$ for 2 min, washed with cold PBS, and suspended in 500 μl IP buffer [20 mM Tris-HCl (pH 7.9), 0.2 mM EDTA, 10% glycerol, 0.2 mM phenylmethylsulfonyl fluoride] with 150 mM KCl and 0.1% Nonidet P-40 (NP-40). Cell lysates were cleared by centrifugation at $20,800 \times g$ for 10 min at 4 C and used for protein interaction studies as described below.

Protein interaction studies

For the *in vivo* interaction studies between endogenous CHD8 and AR, nuclear extracts were prepared from the indicated cell line as described by Dignam *et al.* (61). Nuclear extracts were incubated overnight at 4 C with the specified antibody bound to protein A-agarose beads (Repligen, Waltham, MA). Beads were then washed sequentially with 150 mM KCl in IP buffer containing 0.2% NP-40, 350 mM KCl in IP buffer, 500 mM KCl in IP buffer, 750 mM KCl in IP buffer (three times), 150 mM KCl in IP buffer containing 0.2% NP-40 (twice), and finally in 150 mM KCl in IP buffer. Samples were eluted in sodium dodecyl sulfate (SDS)-loading buffer [125 mM Tris-HCl (pH 6.8), 0.5% SDS, 10% glycerol, 175 mM β-mercaptoethanol] and subjected to SDS-PAGE followed by Western blot analysis with the indicated antibodies. For the interaction studies between recombinant CHD8 and AR, the cleared SF9 cell lysates described above were incubated overnight at 4 C with anti-Flag M2 agarose beads (Sigma). Beads were washed sequentially with 150 mM KCl in IP buffer, 150 mM KCl in IP buffer with 0.1% NP-40, and 150 mM KCl in IP buffer before eluting as described above. Samples were then subjected to SDS-PAGE and Western blot analysis with the indicated antibodies.

ChIP

ChIP experiments were performed essentially as described in the ChIP assay kit (Upstate Biotechnology, Lake Placid, NY). Briefly, approximately 1×10^6 cells per immunoprecipitation were fixed with 2.5% formaldehyde for 10 min at 37 C. Cells were washed with cold PBS and lysed with ChIP lysis buffer. The chromatin was sheared by sonication (~200- to 1000-bp fragments) and cleared by centrifugation at $20,000 \times g$ for 10 min

at 4 C. Samples were diluted 10-fold in ChIP dilution buffer and then precleared with protein A agarose beads (Repligen) blocked with salmon sperm DNA (Invitrogen). Samples were incubated overnight at 4 C with the indicated antibody. Chromatin-antibody complexes were precipitated by incubation with protein A agarose beads blocked with salmon sperm DNA. Samples were then washed and eluted as described in the instructions, except washes were done for 15 min each. For re-ChIP experiments, the first ChIP was done as described above except the washed chromatin-antibody complexes were eluted in 50 μl Tris-EDTA with 10 mM dithiothreitol. Samples were then diluted 20-fold in re-ChIP buffer [150 mM NaCl, 0.5 mM dithiothreitol, 1% Triton X-100, 20 mM Tris-HCl (pH 8.1), 2 mM EDTA] and immunoprecipitated with the second antibody, washed, and eluted as per the standard ChIP protocol.

RT-PCR and quantitative PCR

cDNA was prepared by extracting total RNA from the indicated cells using the RNeasy kit (QIAGEN, Valencia, CA) following the manufacturer's instructions. Reverse transcription reactions employed total RNA, random decamers (Ambion, Austin, TX), and Superscript II reverse transcriptase (Invitrogen) following the manufacturers' instructions. Real-time quantitative analysis employed the indicated primers, iQ SYBR Green Supermix (Bio-Rad, Hercules, CA), and a MyiQ single-color real-time PCR detection system (Bio-Rad). All real-time PCR were performed in triplicate. For RNA expression analysis, threshold cycle values were normalized to levels of RNA polymerase III-transcribed human H1 RNA. For ChIP experiments, DNA quantities were expressed relative to input levels. The following primers were used for ChIP experiments: TMPRSS2 −13.5 kb, TGGTCCTGGATGATAAAAAAAGTTT and GACATACGCCCCACAACAGA, and TMPRSS2 −7 kb, ACGCCTTCGCTGTCCTACCT and TGCAATGAAGTTCCCTGCAA. The following primers were used for quantitative RT-PCR analysis: TMPRSS2, GGACAGTGTGCACCTCAAAGA and TTGCTGCCCATGAACTTCC, and H1 control, ACTCCACTCCCATGTCCCTTG and CCGTTCTCTGGGAACTCACCT.

RNA interference knockdown experiments

RNA interference experiments in the various prostate cancer cell lines used the UI2-Puro-SIBR siRNA vectors (44). CHD8 was knocked down using two siRNA cassettes cloned into the UI2-Puro-SIBR vector (26). Vectors containing an siRNA cassette targeting GFP were used as a control. Constructs were transfected into cells in either six-well plates (for expression studies) or in 10-cm dishes (for ChIP) using Lipofectamine 2000 (Invitrogen) as described by the manufacturer. Transfections were done in phenol red-free RPMI 1640 medium containing 10% dextran/charcoal-stripped FBS. After 24 h, the cells were selected with 10 μg/ml puromycin and grown for an additional 48 h. Cells were then treated with DHT or ethanol for 6 h. Efficacy of knockdown was verified by Western blot analysis.

Cell proliferation assays

LNCaP cells were transfected and selected as described above. The cells were washed with PBS, trypsinized, and counted. The cells were then plated in 96-well plates at 5×10^3 cells per well and treated with either 4 nM R1881 or vehicle. Cells were harvested at the specified time point, and the cell growth was measured using the CellTiter-Glo luminescent cell viability assay (Promega) following the manufacturer's instructions.

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